



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/31, 15/54, 15/55, C07K 14/315, C12N 8/22, 8/10, 15/75, 1/20, A23C 8/123 // (C12N 1/20, C12R 1:46)	A2	(11) International Publication Number: WO 96/21017 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/NL95/00448 (22) International Filing Date: 29 December 1995 (29.12.95) (30) Priority Data: 08/366,480 30 December 1994 (30.12.94) US 08/424,641 19 April 1995 (19.04.95) US (71) Applicant (for all designated States except US): QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): MOINEAU, Sylvain [CA/CA]; 3605 Place du Houx, Charlesbourg, Quebec G1G 3G9 (CA). WALKER, Shirley, A. [US/US]; Apart- ment 16, 5416 Portree Place, Raleigh, NC 27606 (US). VEDAMUTHU, Ebenezer, R. [US/US]; 2710 5th Avenue N.W., Rochester, MN 55901 (US). VANDENBERGH, Peter, A. [US/US]; 4414 Meadowcreek Circle, Sarasota, FL 33583 (US). (74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibu- reau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: ISOLATED DNA ENCODING ENZYME FOR PHAGE RESISTANCE (57) Abstract An isolated DNA of a <i>Lactococcus lactis</i> showing a SEQ ID NO:1 encoding a restriction and two modification enzymes (R/M SEQ ID NO:2, 3 and 4). The isolated DNA is used to transform sensitive dairy cultures, such as <i>Lactococcus lactis</i> and <i>Streptococcus thermophilus</i> , to provide phage resistance. <i>Escherichia coli</i> can be used to produce endonucleases.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

ISOLATED DNA ENCODING ENZYME FOR PHAGE RESISTANCE

BACKGROUND OF THE INVENTION(1) Field of the Invention

The present invention relates to transformed dairy cultures with a natural 7.8-kb plasmid pSRQ700 which was isolated from *Lactococcus lactis* subsp. *cremoris* DCH-4, a known strain. pSRQ700 encodes a restriction/modification system named *LlaII*. When introduced into a phage-sensitive dairy culture, such as *L. lactis*, pSRQ700 confers strong phage resistance against the three most common lactococcal phage species: 936, c2 and P335 found in dairy product fermentations. The *LlaII* endonuclease was purified and found to cleave the palindromic sequence 5'/GATC-3'. The low copy plasmid pSRQ700 was mapped and the genetic organization of *LlaII* localized. Cloning and sequencing of the entire *LlaII* system allowed the identification of three open reading frames. The three genes (*LlaIIA*, *LlaIIB*, and *LlaIIC*) overlapped and are under one promoter. A terminator was found at the end of *LlaIIC*. The genes *LlaIIA* and *LlaIIB* coded for m⁶A-methyltransferases and *LlaIIC* for an endonuclease. The native *LlaII* R/M system from *Lactococcus lactis* is also expressed by and conferred strong phage resistance to various industrial *S. thermophilus* strains. Resistance was observed against phages isolated from yogurt and Mozzarella wheys. This is the first demonstration of increased phage resistance in *S. thermophilus*.

(2) DESCRIPTION OF RELATED ART

Lactococcus lactis and *Streptococcus salivarius* subsp. *thermophilus* cultures are used extensively worldwide in the manufacture of fermented dairy products. The cultures are normally inoculated into pasteurized or heat-treated milk to quickly start and control the fermentation. In this non-sterile milk environment, the added cells come into contact with the wild bacteriophage population that has survived pasteurization. Although natural phage concentration is low, their population increases very rapidly if phage-sensitive cells are present in the starter culture. The consequent lysis of a large number of sensitive cells retards the fermentation process. To cope with this natural phenomenon, the dairy industry has developed a series of solutions including the use of phage resistant *Lactococcus lactis* strains (Hill,

3C., FEMS Microbiol. Rev. 12:87-108 (1993)). Lactococcus lactis

In the last decade, extensive research was conducted on interactions between lactococcal phage and their hosts. *Lactococcus lactis* was found to possess many plasmids coding for natural defense mechanisms against bacteriophages. Over 40 plasmids with phage defense barriers have been identified. Phage resistance systems are classified into three groups based on their mode of action: blocking of phage adsorption, restriction/modification and abortive infection. Phage-resistant *Lactococcus lactis* strains have been constructed by introducing these natural plasmids into phage-sensitive strains (Sanders, M. E., et al., Appl. Environ. Microbiol. 40:500-506 (1980)). The conjugative abilities of some of these plasmids was exploited to construct phage-resistant strains (Harrington, A., et al., Appl. Environ. Microbiol. 57:3405-3409 (1991); Jarvis, A. W., et al., Appl. Environ. Microbiol. 55:1537-1543 (1988); Sanders, M. E., et al., Appl. Environ. Microbiol. 52:1001-1007 (1986); and Ward, A. C., et al., J. Dairy Sci. 75:683-691 (1992)). However, after considerable industrial use of these strains, new phages capable of overcoming the introduced defense mechanism have emerged (Alatossava, T., et al., Appl. Environ. Microbiol. 57:1346-1353 (1991); Hill, C., et al., J. Bacteriol. 173:4363-4370 (1991); and Moineau, S., et al., Appl. Environ. Microbiol. 59:197-202 (1993)). Thus, the search for different natural phage barriers is still an ongoing objective for dairy product starter culture manufacturers.

Over the years several studies have established the heterologous nature of the lactococcal phage population (Jarvis, A. W., et al., Intervirology 32:2-9 (1991)). Based on electron microscopy and DNA hybridization studies, the Lactococcal and Streptococcal Phage Study Group, which is part of the International Committee on Taxonomy of Viruses, reported the existence of 12 different lactococcal phage species. Recently, this number has been reduced to 10 due to the reclassification of the 1483 and T187 species into the P335 species. Strong DNA homology is observed among members of the same species but no homology is found between species (Braun, V., et al., J. Gen. Microbiol. 135:2551-2560 (1989); Jarvis, A. W., et al., Intervirology, 32:2-9 (1991); Moineau, S., et al., Can. J. Microbiol. 38:875-882 (1992); Powell, I. A., et al., Can. J. Microbiol. 35:860-866 (1989); and Prevots, F., et al., Appl. Environ. Microbiol. 56:2180-2185 (1990)). Although many species have been isolated, only three appear to be very problematic for the dairy industry. The species 936 (small isometric head) and c2

(prolate head) have been, by far, the most disturbing lactococcal phage species worldwide. Interestingly, phages from the P335 species (small isometric head) are now being isolated with increasing frequency from North American dairy plants (Moineau, S., et al., Appl. Environ. Microbiol. 59:197-202 (1993)). Two recent surveys revealed that 100% of the 45 lactococcal phages isolated from Canadian cheese plants and U.S. buttermilk plants were classified within one of these three species: 22 phages belonged to the 936 species, 18 to the c2 species and 5 to the P335 species (Moineau, S., et al., J. Dairy Sci. 77:18 suppl. 1 (1994); and Moineau, S., et al., Can. J. Microbiol. 38:875-882 (1992)). Therefore from a practical point of view, industrial *Lactococcus lactis* strains and in general any bacterium to be used as dairy culture should at least be resistant to the three most common phage species: 936, c2 and P335. Due to the diversity of lactococcal phages, the need for phage defense mechanisms with broad activity (attacking many species) is becoming more meaningful. Because of the characteristics of phages, restriction/modification (R/M) systems have the potential to fulfill this objective.

The phenomenon of R/M was first reported more than 40 years ago (Luria, S. E., et al., J. Bacteriol. 64:557-569 (1952)) and received a molecular explanation ten (10) years later (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); and Dussoix, D., et al., J. Mol. Biol. 5:37-49 (1962)). The main biological activity of R/M is believed to be in preventing the entrance of foreign DNA (including phage DNA) into the cell. These gatekeepers are roughly the prokaryotic equivalent of the immune system (Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991)). There are currently more than 2400 known restriction enzymes and over 100 have been cloned and sequenced (Raschke, E., GATA 10:49-60 (1993); and Roberts, R. J., et al., Nucleic Acid Res. 21:3125-3137 (1993)). There are several kinds of R/M systems and they appear to have equivalent biological activities that are however achieved in different ways. At least four types of R/M systems have been identified: I, II, IIs, and IIII (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)). Of these, type II is the simplest and the most common. Illustrative patents are European Patent Application 0 316 677, European Patent Application 0 452 224, U.S. Patent Nos. 4,530,904 to Hershberger, et al., 4,883,756 to Klaenhammer et al., 4,931,396 to Klaenhammer et al and 5,019,506 to Daly et al.

Many R/M systems have been characterized at the protein level. Restriction enzymes are very dissimilar, suggesting an independent evolution and not development from a common ancestor (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)). In contrast, extensive similarities occur among the methyltransferases (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); Klimasauskas, S., et al., Nucleic Acids Res. 17:9823-9832 (1989); Lauster, R., J. Mol. Biol. 206:313-321 (1989); McClelland, M., et al., Nucleic Acids Res. 20:2145-2157 (1992); Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)). They can be grouped into three classes corresponding to the modification types: m⁴C, m⁵C and m⁶A (Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)). m⁴C and m⁶A can be further divided in two (α and β) and three (α , β , and γ) subclasses respectively, based on their amino acid sequences (Klimasauskas, S., et al., Nucleic Acids Res. 17:9823-9832 (1989); and Lauster, R., J. Mol. Biol. 206:313-321 (1989)).

A number of plasmids encoding R/M have been identified in *Lactococcus* (Hill, C., FEMS Microbiol. Rev. 12:87-108 (1993)). Surprisingly, only a handful have been partially characterized. The LlaI R/M system encoded on the conjugative plasmid pTR2030, isolated from *Lactococcus lactis* subsp. *lactis* ME2, was the first to be analyzed at the sequence level (Hill, C., et al., J. Bacteriol. 173:4363-4370 (1991)). The methylase gene of pTR2030 system has been sequenced and the deduced protein was found to share similarities with the type-III methyltransferase (m⁶A), M. FokI (Hill, C. L., et al., J. Bacteriol. 173:4363-4370 (1991)). The endonuclease genes have also been sequenced and four open reading frames were identified (O'Sullivan, D. J., et al., FEMS Microbiol. Rev. 12:P100 (1993)). Recent data have provided evidence for a new class of multisubunit endonucleases (O'Sullivan, D. J., et al., FEMS Microbiol. Rev. 12:P100 (1993)). The restriction complex, however, has yet to be purified and its recognition sequence is unknown.

ScrFI was the first classical type II restriction enzyme isolated from *Lactococcus lactis* and is the only one commercially available (Fitzgerald, G. F., et al., Nucleic Acid Research. 10:8171-8179 (1982)). ScrFI recognizes the sequence 5'-CCN GG-3' where N is any nucleotide. Two methylase genes from the *Lactococcus lactis* subsp. *lactis* UC503 chromosome have been cloned and sequenced (Davis, R., et

al., Appl. Environ. Microbiol. 59:777-785 (1993); and Twomey, D. P., et al., Gene 136:205-209 (1993)). They both coded for a m^5C MTase. The endonuclease gene has yet to be identified. Mayo et al (Mayo, B., et al., FEMS Microbiol. Lett. 79:195-198 (1991) isolated a type II
5 endonuclease (also named *LlaI*) from *L. lactis* subsp. *lactis* NCD0497 which recognized the sequence 5'-CCWGG-3' (W is A or T) but the R/M genes have not been cloned.

Recently Nyengaard, N., et al, Gene 136, 371-372 (1993) described *LlaI* and *LlaBI*, which are type II restriction endonucleases
10 from *Lactococcus lactis* subsp. *cremoris* W9 and W56. These endonucleases recognize DNA sequences 5'/GATC-3' and 5'-C/TRYAG3', respectively. The plasmids from these strains were transformed into a plasmid free and endonuclease negative *Lactococcus lactis* subsp. *lactis* by electroporation to produce a transformed strain which resisted phage attack. The DNA was
15 not isolated and sequenced and the natural plasmid was used for the transformation. Further, the authors did not indicate if the plasmids encoded methyl transferase. Strains W9 and W56 were not tested. In Journal of Bacteriology, July 1991, p 4363-4370 Hill C. et al. describe the *LlaI* as being a protein of 72.5 kDa with organisational similarities
20 to the type IIa methylase FokI. It is atypical of other type II proteins which generally have a molecular weight of 30-50 kDa. The use of such a sequence for rendering lactobacilli or streptococci resistant to the large group of the three most common phage species: 936, c2 and P335 is not disclosed. They merely describe a plasmid comprising the nucleic acid
25 encoding the 421 amino acids of the amino domain of a truncated protein and illustrated this was sufficient to encode a functional methylase enzyme.

Streptococcus thermophilus

Similar information on phage and phage resistance is still very
30 limited for *Streptococcus thermophilus* despite sustained phage infections in the yogurt and Mozzarella cheese industry (Mercenier et al, Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus*, In M. J. Gasson and W. M. DeVos (ed.), Genetics and biotechnology of lactic acid bacteria. Blackie Acad. Prof. Glaskow, UK p. 253-293 (1994)).
35 Fortunately, *S. thermophilus* phages are much more closely related to each other than the *L. lactis* phages. It appears that there is only one *S. thermophilus* phage species (Mercenier et al Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus*, In M. J. Gasson and W. M. DeVos (ed.), Genetics and biotechnology of lactic acid

bacteria. Blackie Acad. Prof. Glaskow, UK p. 253-293 (1994)). Only very few phage defense mechanisms have been reported for *S. thermophilus*. Four chromosomally-encoded type II R/M systems have been identified in *S. thermophilus*. Solaiman and Somkuti (Solaiman, D.K.Y., et al., FEMS Microbiol. Lett. 67:261-266 (1990); and Solaiman, D.K.Y., et al., FEMS Microbiol. Lett. 80:75-80 (1991)) have isolated the endonuclease Sth134I and Sth117I which are isoschizomers of HpaII and EcoRII, respectively. Benbadis et al (Benbadis, L., et al., Appl. Environ. Microbiol. 57:3677-3678 (1991)) and Guimont et al (Guimont, C., et al., Appl. Microbiol. Biotechnol. 39:216-220 (1993)) have isolated the endonucleases sslI and Sth455I, respectively. Both are also isoschizomers of EcoRII. In addition, *S. thermophilus* might possess abortive-like phage defense mechanisms (Larbi et al. J. Dairy Res. 59:349-357 (1992)), although definitive proof has yet to be demonstrated. None of the R/M systems so far identified in *S. thermophilus* have been cloned, sequenced, or used in commercial strains for improvement of phage resistance. There is believed to be no report on improvement of phage resistance of *S. thermophilus* strains.

OBJECTS

It is therefore an object of the present invention to provide an isolated DNA encoding only restriction and modification enzymes to impart phage resistance. Further, it is an object of the present invention to provide transformation vectors and transformed bacteria incorporating the DNA which are particularly useful in the dairy industry. These and other objects will become increasingly apparent by reference to the following description and the drawings.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is an electrophoresis gel showing a plasmid analysis of *Lactococcus lactis* strains wherein Lane 1 is supercoiled DNA ladder (GIBCO/BRL); Lane 2 is *Lactococcus lactis* DCH-4; Lane 3 is *Lactococcus lactis* SMQ-17 (pSA3 and pSRQ700); Lane 4 is *Lactococcus lactis* SMQ-16 (pSA3).

Figure 2 is an endonuclease restriction map of lactococcal plasmid pSRQ700. Site positions are indicated in kb.

Figure 3 is a map showing cloning of *LlaII* from pSRQ700 into pSA3. Clones were electroporated into LM0230. Transformants were tested for phage resistance against ϕ p2.

Figure 4 is a nucleotide sequence of the 3-kb *NruI*-*EcoRI* fragment from pSRQ700. The deduced amino acid sequence of the 3 ORFs is

presented. The putative promoter, terminator and ribosome binding site are underlined. The first codon of each ORF is in bold. The amino acids are in single letter code.

Figure 5 is a chart showing a comparison of the amino acids between A) M.M. *LlaIIA* (SEQ ID NO. 2), M. *DpnII* (SEQ ID NO. 5), M. *MboA* (SEQ ID NO. 6) and *E. coli* *Dam* (SEQ ID NO. 7) methylases; B) M. *LlaIIB* (SEQ ID NO. 3), *DpnA* (SEQ ID NO. 8), M. *MboC* (SEQ ID NO. 9) and M. *HinfI* (SEQ ID NO. 10); C) R. *LlaII* (SEQ ID NO. 4), R. *DpnII* (SEQ ID NO. 11) and R. *MboI* (SEQ ID NO. 12). The asterisk (*) indicates conserved amino acids. Bars show gaps in the aligned sequences.

Figure 6 is an electrophoresis gel showing restriction patterns of $\phi Q1$, $\phi Q3$ and $\phi Q5$. Lane 1 and 5, 1-kb ladder (Bethesda Research Laboratories); Lane 2, $\phi Q1$ DNA cut with *EcoRV*; Lane 3, $\phi Q2$ cut with *EcoRV*; Lane 4, $\phi Q5$ cut with *EcoRV*; Lane 6, $\phi Q1$ cut with *MboI*; Lane 7, $\phi Q7$ cut with *MboI*; Lane 8, $\phi Q5$ cut with *MboI*.

Figure 7 is a schematic flow sheet showing the construction of the plasmids used in this study.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention relates to an isolated nucleic acid encoding only a protein, polypeptide or enzyme which is sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* and mixtures thereof to restrict or modify a phage. The nucleic acid according to the invention does not comprise the nucleic acid sequence encoding the amino domain of the truncated *LlaII* protein described in Journal of Bacteriology 1991 already cited as this is a *LlaI* derivative and not a *LlaII* derivative. A *LlaI* protein is approximately 72,5 kDa. A *LlaII* protein is less than 70 kDa, generally between 30 and 60 kDa, most preferably between 30 and 50 kDa. Preferably the nucleic acid sequence according to the invention is considered food grade i.e. is derived from a food grade organism or encodes a product that occurs in a food grade organism. Suitable food grade organisms are organisms used in dairy culture. A suitable organism from which a nucleic acid sequence according to the invention can be derived is a Lactobacillus or a Streptococcus.

Preferably the expression product of a nucleic acid sequence according to the invention exhibits the phage restriction or modification activity under circumstances present during dairy processing. Preferably a nucleic acid sequence according to the invention will correspond to a naturally occurring sequence in a food grade organism.

The nucleic acid according to the invention encodes a protein, polypeptide or enzyme wherein "sufficiently duplicative" implies having activity selected from m⁶A methyl transferase activity and endonuclease activity. Suitably the endonuclease activity of the "sufficiently duplicative" protein, polypeptide or enzyme is directed at the palindromic recognition site 5'/GATC-3.' A polypeptide, protein or enzyme that can be considered sufficiently duplicative of LlaII will generally be less than 70 kDa. A protein, polypeptide or enzyme that can be considered "suitably duplicative" is any expression product of an allelic derivative of the encoding nucleic acid sequences ORF 1, ORF 2 and ORF 3 of Sequence Id. No. 1 and sequence Id. Nos 2, 3 and 4 which expression product exhibits the aforementioned phage restriction or modification activity. In particular the expression product may exhibit m⁶A α or β methyl transferase or endonuclease activity or a combination thereof. Any nucleic acid sequence encoding an amino acid sequence encoded by any of the ORF 1, ORF 2 and ORF 3 nucleic acid sequences of Sequence id no. 1 or Sequence id nos 2-4 is included within the scope of protection by virtue of the term "suitably duplicative" on the basis of the degeneracy of the genetic code. Modified nucleic acid sequences of ORF 1, ORF 2 and ORF 3 or Sequence id nos 2-4 encoding expression products with essentially the same degree of phage restriction or modification activity as the expression products of ORF 1, ORF 2 and ORF 3 of Sequence id. no. 1 or Sequence id nos 2-4 or even better activity are also considered "sufficiently duplicative" within the terms of the invention.

In a specific embodiment the present invention relates to an isolated nucleic acid with a nucleotide sequence essentially corresponding to that set forth in SEQ ID NO. 1 selected from the group consisting of ORF1 (positions 97 to 948), ORF2 (positions 941 to 1747) and ORF3 (positions 1740 to 2651) or Sequence id nos 2-4 and combinations thereof. Any nucleic acid sequence capable of hybridising to any of the aforementioned group of sequences under stringent hybridisation conditions, said nucleic acid sequence further encoding an expression product capable of exhibiting phage restriction or modification activity is included within the term "essentially corresponding" and thus is considered "substantially duplicative". A definition of stringent hybridisation conditions can be found in molecular cloning handbooks and other food related patent applications of applicant and is well known to persons skilled in the art (Maniatis, T., E.F. Fritsch and J. Sambrook 1982, Molecular Cloning: a laboratory manual, Cold Spring Harbor

Laboratory, Cold Spring Harbor, N.Y.).

The coding nucleic acid of SEQ ID NO:1 can have modifications in sequence and still be sufficiently homologous to still encode enzymes which have the necessary phage resistance properties. Generally within
5 75-100% homology is sufficient to be considered "substantially duplicative".

The isolated nucleic acid according to the invention is preferably operatively linked to promoter and/or enhancer sequences such that expression of the polypeptide, protein or enzyme or combination of
10 the polypeptides, proteins or enzymes encoded by the nucleic acid sequence is possible in a host cell. In particular a nucleic acid sequence operatively linked such that it is capable of being expressed in a host cell used in dairy cultures (such as Lactobacilli and Streptococci) forms a preferred embodiment of the invention. The phage to
15 be modified or restricted is preferably a phage that occurs in dairy cultures. In particular a phage belonging to any of the categories of lactococcal phages 936, c2 or p335 is to be restricted or modified by the expression products of the nucleic acid according to the invention. Most preferably the phages to be restricted or modified fall within one of the
20 categories 936 or p335.

The present invention also relates to a plasmid containing nucleic acid encoding an enzyme sufficiently duplicative of a member selected from the group consisting of LlaIIA, LlaIIB and LlaIIC and mixtures thereof to restrict or modify a phage i.e. a plasmid comprising
25 nucleic acid sequence according to the invention as described above falls within the scope of the invention. The plasmid according to the invention is preferably capable of expression of the nucleic acid sequence. In particular said plasmid is capable of expression of the nucleic acid sequence in a host cell used in dairy cultures such host cell for example
30 being a Lactobacillus or Streptococcus. The plasmid according to the invention can be a recombinant plasmid or an isolated naturally occurring plasmid. Preferably the plasmid according to the invention will be food grade. The phage to be modified or restricted is preferably a phage that occurs in dairy cultures. In particular a phage belonging to any of the
35 categories of lactococcal phages 936, c2 or p335 is to be restricted or modified by the expression product(s) of the plasmid according to the invention, with a preference for phages in the category 936 or p335.

Further the present invention relates to a recombinant bacterium harboring a nucleic acid sequence and/or a plasmid containing

the nucleic acid sequence according to the invention as described above. In particular a recombinant bacterium harboring a nucleic acid sequence encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* and mixtures thereof such that upon expression of the nucleic acid the bacterium can restrict or modify a phage falls within the scope of the invention. Recombinant bacteria capable of expressing the nucleic acid sequence under conditions present in dairy processes are preferred. A preferred group are recombinant bacteria where the non recombinant bacteria is useful in dairy processes but is not resistant to a phage that occurs in dairy processing prior to incorporation of the nucleic acid according to the invention. A recombinant bacterium according to the invention can quite suitably comprise a recombinant plasmid according to the invention.

A surprising embodiment of the invention is thus an improved recombinant *Streptococcus*, in particular a *Streptococcus thermophilus*, said improvement residing in the presence of a natural plasmid comprising the natural *Lactobacillus* *LlaII* R/M system as disclosed herein. Naturally an improved *Streptococcus* according to the invention may comprise any of the nucleic acid sequences according to the invention or plasmids according to the invention as disclosed above in various suitable and preferred embodiments for rendering the *Streptococcus* phage resistant.

In particular the present invention relates to a recombinant bacterium, preferably isolated and purified, selected from the group consisting of *Streptococcus salivarius* subsp. *thermophilus* and *Lactococcus lactis* naturally lacking in phage resistance which bacterium contains a heterologous nucleic acid sequence encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* and combinations thereof to impart phage resistance. In particular an embodiment wherein furthermore the nucleic acid sequence for the member is essentially as set forth in any of ORF 1, ORF 2 and ORF 3 of SEQ ID NO:1 or Sequence Id nos 2-4 to impart phage resistance is included. In general a *Streptococcus* suitable for use in food processes, in particular dairy processes can be improved by rendering it phage resistant through incorporation of a heterologous nucleic acid sequence, said heterologous nucleic acid sequence encoding an endonuclease and optionally one or more methyltransferases. The heterologous nucleic acid sequence can encode enzymes with an amino acid sequence derived from a R/M phage resistance system of lactobacilli such

as *Lactococcus lactis*. It is possible to incorporate a naturally occurring R/M plasmid from *Lactobacillus* in a *Streptococcus* to achieve a phage resistant *Streptococcus*.

Further still, the present invention relates to a recombinantly produced purified protein, polypeptide or enzyme containing a sequence of amino acids "sufficiently duplicative" of that of a member selected from the group consisting of ORF 1, ORF 2 and ORF 3 and combinations thereof in SEQ ID NO. 2, 3 or 4 such that restriction or modification of a phage can be performed with the enzyme. In particular a suitable embodiment is a protein or polypeptide that has been produced from isolated nucleic acid corresponding to that of the SEQ ID NO:1. The protein or polypeptide can be used for assays as described hereinafter. Preferably the recombinant protein or polypeptide will exhibit a larger homology at amino acid level than is illustrated by ORF 1, ORF 2 and ORF 3 with the amino acid sequences of Figure 5 i.e. the amino acid sequences of M. DpnII, M. MboA, Dam, M. DpnA, M. MboC, M. HinfI, R. DpnII and R. MboI. The recombinant polypeptide, protein or enzyme will exhibit m⁶A methyl transferase activity and/or endonuclease activity, preferably at the palindromic sequence 5'\GATC-3'.

The recombinant polypeptide, protein or enzyme with m⁶A α methyltransferase activity according to the invention will preferably possess more than 88,9 % amino acid homology with the amino acid sequence of the m⁶A α methyl transferase DpnII as indicated in Figure 5A for M. LlaIIA. The recombinant polypeptide, protein or enzyme with m⁶A α methyltransferase activity will possess at least the consensus sequence indicated in Figure 5A for M. LlaIIA if it is to be considered "sufficiently duplicative" of LlaIIA. Preferably the degree of conserved amino acids will be higher than 20%. The degree of conserved tryptophan residues will be higher than 50%, preferably higher than 70%.

The recombinant polypeptide, protein or enzyme with m⁶A β methyltransferase activity will preferably possess more than 75,4 % amino acid homology with the amino acid sequence of the m⁶A β methyl transferase DpnII as indicated in Figure 5B for M. LlaIIB if it is to be considered "sufficiently duplicative" of LlaIIB. The recombinant polypeptide, protein or enzyme with m⁶A β methyltransferase activity will possess at least the consensus sequence indicated in Figure 5B for M. LlaIIB. Preferably the degree of conserved amino acids will be higher than 28%. The degree of conserved tryptophan residues will be higher than 50%, preferably higher than 70%.

The recombinant polypeptide, protein or enzyme with endonuclease activity will preferably possess more than 31% amino acid homology with the amino acid sequence of the MboI endonuclease and more than 34% amino acid homology with the amino acid sequence of DpnII as indicated in Figure 5C for R. LlaII if it is to be considered "sufficiently duplicative" of LlaIIC. The recombinant polypeptide, protein or enzyme with endonuclease activity will possess at least the consensus sequence indicated in Figure 5C for R. LlaII.

Suitably such enzymatic activity will be exhibited under dairy processing conditions. In particular the activities will be exhibited at temperatures of at least 30°C and can be exhibited at 38°C. Note that nucleic acid sequences, plasmids and recombinant bacteria comprising such nucleic acid sequences as heterologous nucleic acid sequences, where the nucleic acid sequences encode a recombinant protein, polypeptide or enzyme according to the invention are also included within the scope of the invention.

Further, the present invention relates to a method of imparting phage resistance to a bacterium which is sensitive to the phage which comprises incorporating nucleic acid according to the invention .g. recombinant DNA encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of LlaIIA, LlaIIB and LlaIIC and mixtures thereof into the bacterium to impart the phage resistance. Preferably a nucleic acid sequence encoding an expression product with m6 methyltransferase activity and an expression product with endonuclease activity is incorporated. More preferably the methyltransferase activity being m6 A methyl transferase activity, with preference for both α and β activity. The endonuclease activity is directed against GATC. Suitably the nucleic acid sequence encoding the member is that contained in strain *Lactococcus lactis* SMQ-17 deposited as NRRL-B-21337. Preferably the bacterium is a dairy culture.

Finally, the present invention relates to a method for fermenting a dairy product, the improvement which comprises using a dairy culture of bacteria (for example selected from the group consisting of *Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus*) in which a nucleic acid sequence according to the invention is present or is incorporated such that it can be expressed in the fermentation process in a manner known per se for the fermentation process, said nucleic acid sequence encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of LlaIIA,

LlaIIB and *LlaIIC* to impart phage resistance. In general terminology said nucleic acid sequence encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of m6A α methyl transferase, m6A β methyl transferase and GATC endonuclease in any of the embodiments according to the invention disclosed above for the nucleic acid sequences and/or recombinant polypeptides. A particular embodiment comprises application of a dairy culture of bacteria in a fermentation process wherein the nucleic acid sequence imparting the phage resistance is that contained in strain *Lactococcus lactis* SMQ-17 deposited as NRRL-B-21337. The nucleic acid sequence can be introduced in a manner known per se for incorporating nucleic acid sequences in such types of bacterium e.g. using general transformation protocols.

The DNA of SEQ ID NO:1 and Figure 4 (Appendix I) is contained in *Lactococcus lactis* SMQ-17 deposited under the Budapest Treaty on September 29, 1994 as NRRL-B-21337. The strain is available upon request by name and deposit number. The isolated DNA can be obtained by means of EcoRV or NruI-TcoRV digestion of pSRQ700 as described hereinafter.

The art of DNA isolation and cloning is well known to those skilled in the art. Further, the terminology of this art is well developed, see for instance EP 0316677 A2. As used herein, the term "transformed" means to transfer DNA from one bacterium to another in related bacterium. The term "recombinant" as used herein means nucleic acid in a form not existing in nature or in an environment it is not normally associated with in nature. In general the recombinant nucleic acid sequence according to the invention contains DNA encoding only one or more of the sequence of amino acids for *LlaIIA*, *LlaIIB* and *LlaIIC* as set forth in SEQ ID NO:1 Sequence id nos 2-4 or substantially duplicative versions thereof. The recombinant enzymes that are claimed are considered to exclude known naturally occurring isolated enzymes exhibiting either m6A methyl transferase activity or endonuclease activity against the restriction site 5'\GATC-3'. The recombinant enzymes resemble the naturally occurring enzymes with regard to their phage restriction or modification activity but can have different physical configurations.

Various shuttle vectors can be used. pSA3 from Dao, M., et al., Applied Environ. Microb. 49:115-119 (1985) was used.

The recombinant bacterium can be for instance *Escherichia coli*, a *Lactococcus* sp. or a *Streptococcus* sp. used in dairy fermentations.

The *E. coli* are used to produce the enzymes of SEQ ID NO:2, 3 and/or 4 which can be used to produce a DNA or RNA probe in a known manner or can be used to produce antibodies to the enzymes in a well known manner for use in assays for the enzymes. Purification of the enzymes can be
5 achieved in a manner known per se using affinity chromatography and/or molecular filtration.

The preferred use of the transformed cultures containing the recombinant DNA of SEQ ID NO:1 is in dairy product fermentations. Such fermentations are well known to those skilled in the art. The preferred
10 strains are transformed *Lactococcus lactis* and *Streptococcus salivarius* sp. *thermophilus* which are used in the dairy product fermentations.

EXAMPLE 1

Bacterial strains, plasmids, and media. The strains and
15 plasmids and enzymes used in this invention are listed in Tables 1 and 2.

Table 1. Bacterial strains, plasmids and bacteriophages

Bacteria, plasmids and phages	Relevant characteristics	Source
5 <i>L. lactis</i> subsp. <i>cremoris</i> DCH-4 UL8	Industrial strain, multiple plasmids, Lac ⁺ Industrial strain, host for P335 phages, Lac ⁺	Invention Moineau, S., et al., Can. J. Microbiol. 38: 875-882 (1992) Invention
10 SMQ-87	UL8 (pSRQ701), Lac ⁺ , Em ^r	
<i>L. lactis</i> subsp. <i>lactis</i> LMO230	Plasmid free, host for 936 and c2 phages, Lac ⁻	McKay, L.L., et al. Appl. Environ. Microbiol. 23: 1090-1096 (1972)
15 SMQ-16 SMQ-17 SMQ-39 SMQ-40 SMQ-50 SMQ-117 SMQ-140	LMO230 (pSA3), Lac ⁻ , Em ^r LMO230 (pSA3, pSRQ700), Lac ⁻ , Em ^r LMO230 (pSRQ701), Lac ⁻ , Em ^r LMO230 (pSRQ702), Lac ⁻ , Em ^r LMO230 (pSRQ703), Lac ⁻ , Em ^r LMO230 (pSRQ704), Lac ⁻ , Em ^r LMO230 (pSRQ706), Lac ⁻ , Em ^r	Invention Invention Invention Invention Invention Invention Invention
<i>E. coli</i> DH5α	Transformation host	GIBCO/BRL (Grand Island, NY) Invention
25 DMQ-149	DH5α(pSRQ708), Ap ^r	
30 Phages φp2 φsk1 φjj50	Small isometric headed, 936 species, 30.5 kb Small isometric headed, 936 species, 28.1 kb Small isometric headed, 936 species, 30.5 kb	L.L. McKay L.L. McKay J. Josephsen, et al., FEMS Microbiol. Lett. 59:161-166 (1989)

(Table 1)

Bacteria, plasmids and phages	Relevant characteristics	Source
$\phi c2$	Prolate headed, c2 species, 20.7 kb	Sanders, M.E., et al., Appl. Environ. Microbiol. 40:500-506 (1980)
$\phi ml3$	Prolate headed, c2 species, 20.2 kb	W.E. Sandine
$\phi eb1$	Prolate headed, c2 species, 19.6 kb	L.L. McKay
$\phi u136$	Small isometric headed, P335 species, 28.8 kb	Moineau, S., et al., Can. J. Microbiol. 38: 875-882 (1992)
$\phi q30$	Small isometric headed, P335 species, 37.0 kb	Moineau, S., et al., J. Dairy Sci. 77:18 Suppl.1 (1994)
$\phi q33$	Small isometric headed, p335 species, 29.6 kb	Moineau, S., et al., J. Dairy Sci. 77:18 Suppl. 1 (1994)

L.L. McKay, University of Minnesota; W.E. Sandine, Oregon State University; Lac, Lactose-fermenting ability; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance.

5

10

15

20

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source
pSA3	Shuttle vector, Cm, Tc, Em, 10.2 kb	Dao, M.L., et al., Appl. Env. Microb. 49:115-119 (1985)
pBS KS (+)	Cloning vector for sequencing, Ap, 2.9 kb	Stratagene
pSRQ700	Resident plasmid of DCH-4, R'/M', 7.8 kb	This study
pSRQ701	7.0-kb <i>EcoRI</i> fragment from pSRQ700 cloned into pSA3, R'/M', Cm', Tc', Em'	This study
pSRQ702	5.3-kb <i>NcoI-EcoRI</i> fragment from pSRQ700 cloned into pSA3, R'/M', Cm ^s , Tc', Em'	This study
pSRQ703	6.6-KB <i>NcoI</i> fragment from pSRQ700 cloned into pSA3, R'/M', Cm', Tc', Em'	This study
pSRQ704	7.8-kb <i>EcoRV</i> fragment from pSRQ700 cloned into pSA3, R'/M', Cm', Tc', Em'	This study
pSRQ706	3.0-kb <i>NruI-EcoRV</i> fragment from pSRQ700 cloned into pSA3, R'/M', Cm', Em'	This study
pSRQ708	3.0-kb <i>NruI-EcoRV</i> fragment from pSRQ700 cloned into pBS, R'/M', Ap'	This study

Ap' ampicillin resistance; Cm' chloramphenicol resistance; Cm^s sensitive to chloramphenicol; Em', erythromycin resistance; Tc' tetracycline resistance; Tc', tetracycline resistance; R'/M', active restriction/active modification enzymes;

Escherichia coli was grown at 37°C in Luria-Bertani (Sambrooke, J., et al., Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). *Lactococcus lactis* strains were grown at 30°C in M17 (Terzaghi, B. E., et al., Appl. Microbiol. 29:807-813 (1975)) supplemented with 0.5% glucose (GM17) or 0.5% lactose (LM17). When appropriate, antibiotics were added as follows: for *E. coli*, 50µg/ml of ampicillin (Ap), 10µg/ml of tetracycline (Tc), and 20µg/ml of chloramphenicol (Cm); for *L. lactis*, 5 µg/ml of erythromycin (Em).

Bacteriophage propagation and assays. Bacteriophages used in this invention are listed in Table 1. Bacteriophages were propagated and titrated by the method of Jarvis (Jarvis, A. W., Appl. Environ. Microbiol. 36:785-789 (1978)). Efficiency of plaquing (EOP) assays were performed as described by Sanders and Klaenhammer (Sanders, M. E., et al., Appl. Environ. Microbiol. 40:500-506 (1980)). Bacteriophages c2, p2, sk1 and jj50 were supplied by T. R. Klaenhammer (North Carolina State University).

DNA Isolation and manipulation. Plasmid DNA from *E. coli* was isolated as described previously (Moineau, S., et al., Appl. Environ. Microbiol. 60:1832-1841 (1994)). Large quantities of *E. coli* plasmid DNA was isolated by using plasmid MIDI or MAXI kit (Qiagen Inc., Chatsworth, CA). Plasmid DNA from *L. lactis* was isolated as described by O'Sullivan and Klaenhammer (O'Sullivan, D. J., et al., Appl. Environ. Microbiol. 59:2730-2733 (1993)). A large quantity of lactococcal plasmid DNA was obtained using the Leblanc and Lee procedure (Leblanc, D. J., et al., J. Bacteriol. 140:1112-1115 (1979)) as modified by Gonzalez and Ku (Gonzalez, C. F., et al., Appl. Environ. Microbiol. 46:81-89 (1983)). Restriction endonucleases (Gibco/BRL, Grand Island, NY) and T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN) were used according to manufacturer's instructions. When needed, DNA fragments were obtained from low-melting agarose using a QIAEX gel extraction kit (Qiagen, Inc., Chatsworth, CA).

Electroporation. *E. coli* was grown, electroporated, incubated, and plated as described previously (Moineau, S., et al., Appl. Environ. Microbiol. 60:1832-1841 (1994)). *L. lactis* was grown in GM17 supplemented with 0.5M sucrose (SGM17) and 1% glycine and electroporated as described by Holo and Nes (Holo, H., et al., Appl. Environ. Microbiol. 55:3119-3123 (1989)). The Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) was set at 25µF and 2.45 kV, and the Pulse Controller was

set at 200Q. Plasmid DNA was mixed with 40ul of cells in a chilled cuvette (0.2 cm). After electroporation, *L. lactis* cells were resuspended in SGM17, incubated for 2 h at 30°C, plated on GM17 supplemented with erythromycin (5µg/ml) and incubated for 2 days at 30°C.

5 Sequencing. The entire *LlaII* system (3 kb *NruI*-*EcoRV* fragment from pSRQ700) was cloned into *E. coli* pBluescript. The resulting clone was named pSRQ708. Nested deletions were made in both orientations from pSRQ708 using the ERASE-A-BASE kit (Promega, Madison, WI). For the first set of deletions, the endonuclease *SstI* was used for protection and *XbaI* 10 was used to start the deletion. The restriction pairs *KpnI*-*DraII* were used to obtain the nested deletions in the other orientation. Plasmid DNA was extracted from the nested clones with QIAGEN and directly used for sequencing. The sequencing reactions were performed using the DYEDEOXY TERMINATOR TAQ sequencing kit for use on the 373A automated DNA 15 sequencing system (Applied Biosystems, Foster City, CA). The T7 and T3 primers were used for annealing.

 Restriction enzyme purification. *L. lactis* SMQ-17 was grown in 2L, concentrated by centrifugation (10,000 rpm, 15 min.) and washed twice in saline. The cells were then resuspended in 30 ml of PME buffer (10 mM 20 NaH_2PO_4 pH 7.4, 0.1 mM EDTA and 10 mM β -mercaptoethanol). Cells were lysed by 15 bursts (30 seconds each followed by one minute rest) with glass beads and a bead beater (BIOSPEC, Bartlesville, OK). After centrifugation to remove cell debris and glass beads, the supernatant was used for ion exchange chromatography. Successive chromatographies were 25 performed on phosphocellulose (Whatman P11, Maidstone, England) and dimethylaminoethyl cellulose (Whatman DE5, Maidstone, England) using a salt gradient in PME buffer. Restriction endonuclease activity was found in the fractions around 0.5 M NaCl. Lactococcal phage ϕ 136 DNA was used as substrate and the digestions were performed at 37°C for 2-4 h using 30 the buffer system #2 from GIBCO/BRL (50 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 50 mM NaCl). DNA samples were analyzed as described by Sambrooke et al in Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) in 0.7% agarose gels in TAE.

 DNA and protein analysis. The DNA sequence was analyzed with 35 DNA strider 1.2. The SwissProt database (release 29, June 1994) was searched for homology to all three *LlaII* amino acid sequences of SEQ ID NO:1.

 Isolation of pSRQ700. For many years, *Lactococcus lactis* subsp. *cremoris* DCH-4 has performed very well during the industrial

buttermilk and sour cream production. One reason for continued good performance is the natural resistance of DCH-4 to lactococcal bacteriophages. One objective of this invention was to identify and transfer the DNA responsible for the phage resistance of DCH-4. The total plasmid DNA of DCH-4 was isolated and co-electroporated with the vector pSA3 into the phage sensitive-plasmid free *L. lactis* LM0230. The latter strain was selected because it can propagate phages from two species, 936 and c2. The DNA ratio of DCH-4:pSA3 used for electroporation was about 10:1. Em-resistant colonies were tested for phage resistance by spot assay ($10^3 - 10^4$ pfu of ϕ p2/spot). A few phage resistant colonies were obtained, analyzed, and found to contain pSA3 and a 7.8 kb low copy plasmid which was named pSRQ700 (Figure 1). The transformant containing pSRQ700 was named *L. lactis* SMQ-17 (NRRL-21337). Plasmid pSRQ700 was also electroporated into *L. lactis* UL8 which can propagate phages from the P335 species. The transformant was named *L. lactis* SMQ-87.

Effectiveness of pSRQ700 on lactococcal phage species. *L. lactis* SMQ-17 and SMQ-87 were tested for phage resistance against a total of 9 phages belonging to 3 species (3 phages/species). Phages p2, sk1 and jj50 were selected as representatives of the 936 species (Table 1). The lactococcal phage species c2 was represented by the phages c2, m13 and eb1. These six phages were individually tested on SMQ-17 and their EOPs are presented in Table 3.

Table 3

Comparison between the efficiency of plaquing of lactococcal phages on *L. lactis* SMO-17 and the number of *Mbol* sites in the phage genome.

	EOP on SMO-17	Number of <i>Mbol</i> sites*
936 species		
φp2	1.7×10^{-6}	11
φsk1	2.5×10^{-6}	9
φjj50	2.0×10^{-6}	10
c2 species		
φc2	1.0×10^{-4}	3
φml3	6.1×10^{-3}	2
φeb1	5.5×10^{-3}	2
P335 species		
φul36	2.7×10^{-7}	13
φQ30	5.2×10^{-6}	12
φQ33	1.3×10^{-7}	15

* Only number of fragments > 0.5 kb were determined.

The new emerging P335 species was represented by the phages ul36, Q30 and Q33. They were tested separately on SMQ-87 and their EOPs are also presented in Table 3. All three 936 phages had similar EOPs in the range of

5 10^{-6} . More variability was observed with the c2 species where EOPs ranged from 10^{-3} to 10^{-4} . The P335 phages were the most affected by pSRQ700 since EOPs of 10^{-7} were observed (Table 3). Identical results were obtained when phage resistance was tested at 21, 30 and 38°C (data not shown). These results indicated that the phage resistance mechanism
10 encoded on pSRQ700 is temperature insensitive.

Identification of the phage resistance mechanism on pSRQ700. Phages capable of overcoming the defense mechanism encoded on pSRQ700 were isolated. These phages had EOPs of 1.0 on *L. lactis* SMQ-17. When these resistant (modified) phages were propagated back on their original
15 host, they became sensitive (restricted) to pSRQ700 at the same previous level (data not shown). This temporary host specific immunity, demonstrates the presence of a classical R/M system encoded on pSRQ700. The R/M system was named *LlaII*.

Isolation of the restriction endonuclease. The non-specific
20 nucleases were removed after ion exchange chromatographies performed on phosphocellulose (Whatman P11) and dimethylaminoethyl cellulose (Whatman DE5) using a salt gradient in PME (10 mM NaH_2PO_4 pH 7.4, 0.1 mM EDTA and 10 mM β -mercaptoethanol) buffer. DNA from the well-characterized lactococcal phage ul36 (Moineau, S., et al., Can. J. Microbiol. 38:875-
25 882 (1992; Moineau, S., et al., Appl. Environ. Microbiol. 59:197-202 (1993); and Moineau, S., et al., Appl. Environ. Microbiol. 60:1832-1844 (1994)) was digested with *LlaII*. The digestions were conducted overnight at 37°C since the R/M encoded on pSRQ700 is temperature-insensitive (up to 38°C). Defined DNA fragments were identified on agarose gels (data
30 not shown). No attempts were made to determine the number of activity units in the collected fractions nor the percentage of recovery from the crude supernatant. Unexpectedly, the restriction patterns obtained corresponded to *MboI* restriction patterns. Attempts to cut pSRQ700 with *MboI* were unsuccessful. It was concluded that the R/M system present on
35 pSRQ700 was similar to the *MboI* system which recognized the sequence 5'-GATC-3' and cleaved it before the guanine.

Mapping of pSRQ700. Single, double and triple digestions were performed with endonucleases to obtain a map of pSRQ700. The results are presented in Figure 2. The following endonucleases did not cut pSRQ700:

ApaI, *AvaI*, *AvaII*, *BalI*, *BamHI*, *HpaI*, *MboI*, *PstI*, *PvuII*, *SalI*, *SmaI*, *SphI*, *XbaI*, *XhoI*.

Localization of the *LlaII* system on pSRQ700. The *LlaII* R/M system was entirely cloned into *E. coli* using the *E. coli*-*L. lactis* shuttle vector pSA3 (Figure 3). Since appropriate unique restriction sites were present on pSA3 and pSRQ700, total plasmid DNA from *L. lactis* SMQ-17 was directly used for cloning. Plasmid DNA from SMQ-17 was digested with selected endonucleases, phenol extracted, ethanol precipitated, ligated and the ligation mixture electroporated in *E. coli* DH5a. This strategy was very effective because expected clones were rapidly obtained. The clones were electroporated into *L. lactis* LM0230 and phage resistance was determined. The relevant clones are presented in Figure 3. The entire R/M system of pSRQ700 was localized on a 3-kb *NruI*-*EcoRV* fragment. The pSA3 clone containing this 3kb fragment was named pSRQ706. Similar EOPs were obtained with pSRQ700 and pSRQ706 (Figure 3). This is due to the similar low copy number of pSA3 and pSRQ700 (Figure 1).

DNA Sequence Analysis of the *LlaII*. The 3-kb *NruI*-*EcoRV* fragment containing the *LlaII* genes was sequenced in both directions and found to contain 2,987 bp (Figure 4; SEQ ID NO:1). This fragment was 65.7% A+T rich, typical of lactococcal genes (Van de Guchte, M., et al., FEMS Microbiol. Rev. 88:73-92 (1992)). Three overlapping open reading frames (orfs) were found and the genes were named *LlaIIA*, *LlaIIB* and *LlaIIC*. In reference to Figure 4 and SEQ ID NO:1, the gene *LlaIIA* was localized from position 97 to position 948 and coded for a protein of 284 amino acids with an estimated weight of 33,031 Da. The gene *LlaIIB* was localized from position 941 to position 1747 and coded for a protein of 269 amino acids with an estimated weight of 30,904 Da. The gene *LlaIIC* was localized from position 1740 to position 2651 and coded for a protein of 304 amino acids with an estimated weight of 34,720 Da. Phage p2 EOP of 1.0 on *L. lactis* harboring pSRQ702 or pSRQ703 suggested that *LlaIIC* coded for the endonuclease (Figure 3). No putative ribosome binding site (RBS) was found for *LlaIIA* and *LlaIIB*. A putative RBS (GGAG) was found preceding *LlaIIC*. Atypical RBS have been identified for the *DpnII* methylases which are similar to *LlaII* (Figure 5). They were not found in the *LlaII* system. Atypical RBS may be related to translational control of the methylase gene expression (Lacks, S. A., et al., In: Genetics and Molecular Biology of Streptococci, Lactococci and Enterococci, Dunny, G. M., P. P. Cleary and L. L. McKay. (eds) ASM, Washington, D. C. p.71-76

(1991)). All three genes appear to be under the control of the same promoter. However, no definite consensus *E. coli*-10 and -35 promoter sequences could be identified. Because EOPs were the same in PSRQ700, pSRQ701 and PSRQ703 (Figure 3), it is believed that the promoter was present in the 3.0-kb fragment. The putative promoter sequences upstream of *LlaIIA* is of interest. A putative -35 region was localized at position 27, followed by a 18 bp spacer, and a putative -10 region at position 51 (Figure 4). A search for palindromic sequences identified two perfect inverted repeats of 19 bp, typical of a strong rho-independent terminator, at the very end of *LlaIIC* (Figure 4). Interestingly, the stop codon of *LlaIIC* was within the beginning of the stemloop structure.

Protein analysis. Homology searches showed that the deduced protein coded by *LlaIIA* was 75.4% identical to *DpnII* methylase (Mannarelli, B. M., et al., Proc. Natl. Acad. Sci. 82:4468-4472 (1985)), 41.5% identical *MboI* methylase (Ueno, T., et al., Nucleic Acids Res. 10:2309-2313 (1993)) and 30.1% to the *Dam* methylase of *E. coli* (Brooks, J. E., et al., Nucleic Acids Res. 11:837-851 (1983)). It was concluded that *LlaIIA* codes for a methylase and was named M.*LlaIIA*. All three methylases (M.*DpnII*, M.*MboA* and *Dam*) homologous to *LlaIIA* are N-6 adenine methyltransferase (m^6A -MTases). The most conserved amino acid sequence motifs among the m^6A -MTases are F-G-G (motif I) and DPPY (motif II). Their organization in the protein allowed the division of the m^6A -MTases in three subclasses (α , β and γ). In the m^6A -MTase subclass α , the motif I is found close to the N-terminal followed by a variable region of 100-200 aa and the motif II close to the C-terminal. The reverse situation is found in the subclass β , where the motif II appears before the motif I. M.*LlaIIA* has all the characteristics of a m^6A -MTase subclass α : F-G-G motif, a 146 aa variable region and a DPPY motif (Figure 5). The F-G-G motif probably contained the S-adenosylmethionine binding site and DPPY might be involved in the methylation of exocyclic amino acids (Klimasauskas, S., et al., Nucleic Acids Res. 17:9823-9832 (1989)).

The deduced protein coded by *LlaIIB* was found to be 88.9% identical to the second methylase of *DpnII* (Cerritelli, S., et al., Proc. Natl. Acad. Sci. USA, 86:9223-9227 (1989)), 50.2% identical to the second methylase of *MboI* (Ueno, T., et al., Nucleic Acids Res. 10:2309-2313 (1993)) and 43.6% identical to the *Hin/I* methylase (Chandrasegaran, S., et al., Gene 70:387-392 (1988)). It was concluded that *LlaIIB* also codes for a methylase and was named M.*LlaIIB*. All three methylases (M.*DpnA*,

M.MboC and HinfI) homologous to LlaIIB are m⁶A-Mtases but subclass β . M.LlaIIB has all the subclass β characteristics: a DPPY motif, a 175 aa variable region and a F-G-G motif. Interestingly, Figure 5 also showed the amino acid comparison between two sets of four m⁶A-Mtases isolated from two Gram-positive and two Gram-negative bacteria. This enzyme methylates the same 5'-GATC-3' sequence. Despite the various origins, about 20% and 28% of the amino acids are respectively conserved in the four α and β methylases studied. Interestingly, almost all tryptophan residues are conserved in the methylases studied (Figure 5).

The deduced protein coded by LlaIIC was 34% and 31% identical to MboI (Ueno, T., et al., Nucleic Acids Res. 10:2309-2313 (1993)) and DpnII (de la Campa, A. G., et al., J. Biol. chem. 263:14696-14702 (1987)) endonucleases, respectively. These results confirmed that LlaIIC coded for an endonuclease and was named R.LlaII. Conserved aa motifs were observed among the three isoschizomers but their functionality is unknown.

It was thus found that *Lactococcus lactis* subsp *cremoris* DCH-4 harbors a 7.8-kb low copy plasmid (PSRQ700) coding for a temperature-insensitive R/M system similar to DpnII (Lacks, S. A., et al., In: Genetics and Molecular biology of Streptococci, Lactococci and Enterococci. Dunny, G. M., P. P. Cleary and L. L. McKay. (eds) ASM, Washington, D. C. p-71-76 (1991)) and MboI (Ueno, T., et al., Nucleic Acids Res. 10:2309-2313 (1993)). These systems recognize the non-methylated DNA sequence 5'-GATC-3' where the endonuclease cleaved before the guanine (Lacks, S. A., et al., In: Genetics and Molecular biology of Streptococci, Lactococci and Enterococci. Dunny, G. M., P. P. Cleary and L. L. McKay. (eds) ASM, Washington, D. C. p-71-76 (1991); and (Ueno, T., et al., Nucleic Acids Res. 10:2309-2313 (1993)). The plasmid PSRQ700 is probably one reason for the strong phage resistance shown by DCH-4 over the years. Any phage containing the non-methylated GATC sequence in its genome will be restricted when infecting a *L. lactis* strain containing PSRQ700.

Members of the three most common lactococcal phage species were strongly restricted by PSRQ700 as shown by their reduced EOPs (Table 3). The small isometric-headed phages of the P335 and 936 species were particularly affected by PSRQ700. This is due in part to their larger genomes. The average genome size for the P335, 936 and c2 phages used in this study was 31.8, 29.7 and 20.2-kb, respectively. However, the most important factor was the number of LlaII sites in the phage genome.

Three *LlaII* sites in the prolate ϕ c2 genome were enough to restrict its EOP by 4 logs on *L. lactis* SMQ-17 (Table 3). Two *LlaII* sites in the ϕ ml3 and ϕ eb1 genomes were still enough to reduce the EOP by 3 logs. These data are in agreement with the single hit kinetic of R/M system and shows that restriction at one site is enough to prevent phage proliferation (Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)). For the small isometric phages which had more *LlaII* sites in their genome, the presence of 9 to 12 sites gave a 6 log reduction in EOP, whereas 13 to 15 sites were needed for a 7 log reduction. As reported previously, the EOP decreases logarithmically as the number of sites in the phage genome increases (Wilson, G. G., et al., Annu. Rev. Genet. 25:585-627 (1991)).

Thus, phage resistance conferred by PSRQ700 was substantial against members of the 3-lactococcal phage species tested.

Close gene linkage is a feature of all R/M system and accordingly *LlaII* genes are adjacent (Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al. Annu. Rev. Genet. 25:585-627 (1991)). The *LlaII* system is highly related to *DpnII* (Lacks, S. A., et al., In: Genetics and Molecular Biology of Streptococci, Lactococci and Enterococci. Dunny, G. M., P. P. Cleary and L. L. McKay. (eds) ASM, Washington, D. C. p. 71-76 (1991)). They share the same genetic structure: two methylases followed by an endonuclease (de la Campa, A. G., et al., J. Mol. Biol. 196:457-469 (1987)). There is also gene overlapping in both systems. The most striking similarity is their methylases (Cerritelli, S., et al., Proc. Natl. Acad. Sci. USA. 86:9223-9227); and Mannarelli, B. M., et al., Proc. Natl. Acad. Sci. 82:4468-4472 (1985)). Amino acids comparison showed 75% identity between M.*LlaIIA* and M.*DpnII* and 88% between M.*LlaIIB* and M.*DpnA* (Figure 5).

Despite the strong homology between *LlaII* and *DpnII* methylases, the endonucleases are still divergent. Only 31% of the amino acids are identical. In fact, the endonuclease of *LlaII* is more homologous to *MboI* than to *DpnII*. One might suggest that the methylase had a common ancestor whereas endonucleases evolved independently (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); (Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al. Annu. Rev. Genet. 25:585-627 (1991)). Many type II R/M system appear to have formed partnerships with miscellaneous genes that were initially separated. They became linked due to a persistent selective advantage (Bickle, T. A., et al., Microbiol. Rev. 57:434-450 (1993); (Wilson, G. G., Nucleic Acids Res. 19:2539-2566 (1991); and Wilson, G. G., et al. Annu. Rev.

Genet. 25:585-627 (1991)).

Finally, from a culture manufacturer standpoint, the introduction of the natural low copy number PSRQ700 into industrial *Lactococcus lactis* strains can confer strong phage resistance against phages of the 936 species and the newly emerging P335 species. Its effectiveness against c2 species will be variable. The temperature insensitivity nature of LlaII (up to 38°C) makes this phage resistance mechanism amenable to various types of high-temperature dairy fermentations, especially cheese. The use of PSRQ700 as part of a starter rotation scheme (to avoid the build up of modified phages) can improve the overall phage resistance of starter cultures.

EXAMPLE 2

The native LlaII R/M system from *Lactococcus lactis* was expressed by and conferred strong phage resistance to various industrial *S. thermophilus* strains. Resistance was observed against phages isolated from yogurt and Mozzarella wheys.

Bacteria, bacteriophages, and media. The strains used in this study are listed in Table 4. *S. thermophilus* strains were confirmed by Rapid ID 32 Strep (BioMérieux Vitek, Inc., Hazelwood, MO). *Streptococcus thermophilus* strains were grown at 42°C in GM17. When needed, antibiotics were added at 5 µg of chloramphenicol per ml. Bacteriophages used in this study are listed in Table 4.

Table 4. Bacteria and bacteriophages used in this study

Bacteria or phage	Relevant characteristics*	Source
<i>E. coli</i> DH5 α	Transformation host	Gibco/BRL
<i>L. lactis</i> LMO230 SMQ-17 SMQ-151	Plasmid-free, Lac ⁻ , host for ϕ p2 LMO230 (pSRQ700) LMO230 (pSRQ707), Cm ^r	38 This invention This invention
<i>S. thermophilus</i> SMQ-119 SMQ-146 SMQ-154 SMQ-173 SMQ-174 SMQ-211 SMQ-212	Industrial strain used in yogurt, host for ϕ Q1 and ϕ Q3 SMQ-119 (pNZ123), Cm ^r SMQ-119 (pRQ707), Cm ^r Industrial strain used for Mozzarella, host for ϕ Q5 Industrial strain used for Mozzarella, host for ϕ Q6 SMQ-211 SMQ-173 (pSRQ707), Cm ^r SMQ-174 (pSRQ707), Cm ^r	This invention This invention This invention This invention This invention This invention This invention
Phages ϕ p2 ϕ Q1 ϕ Q3 ϕ Q5 ϕ Q6	<i>L. lactis</i> phage, small isometric-head, 936 species <i>S. thermophilus</i> phage isolated from yogurt <i>S. thermophilus</i> phage isolated from yogurt <i>S. thermophilus</i> phage isolated from Mozzarella whey <i>S. thermophilus</i> phage isolated from Mozzarella whey	L.L. McKay This invention This invention This invention This invention

L.L. McKay, University of Minnesota; Cm^r, chloramphenicol resistance;
Lac⁻, deficient in lactose fermenting ability.

Streptococcal phages were propagated by the method of Jarvis et al (Jarvis, A.W., et al., Intervirology 32:2-9 (1991)). EOP assays on *S. thermophilus* hosts were performed as follows: strains were grown in GM17 overnight at 37 C, 500µl of cells and 100µl of diluted phages were mixed with 2.5 ml of soft agar (GM17 supplemented with 10mM CaCl₂) and layered onto bottom agar (GM17 + CaCl₂). Plates were incubated overnight 42°C in an anaerobic jar (BBL GasPaK Plus, Beckton Dickinson, Cockeysville, MD).

DNA isolation and manipulation. Plasmid DNA from *S. thermophilus* was isolated by using the method of O'Sullivan and Klaenhammer (O'Sullivan, D. J., et al., Appl. Environ. Microbiol. 59:2730-2733 (1993)). Phage DNA was isolated as described previously (Moineau, S., et al., Appl. Environ. Microbiol. 60:1832-1841 (1994)).

Electroporation. *S. thermophilus* cells were electroporated as follows: cells were grown in GM17 until mid-exponential phase, centrifuged, washed twice with SG buffer (0.5M sucrose and 10% glycerol) and put on ice until use. Plasmid DNA (1µg) was mixed with 40 µl of cells in a chilled Gene Pulser cuvette (0.2 cm). The Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) was set at 25 µF and 2.45 kV, and the Pulse Controller at 200 Ω. After electroporation, the *S. thermophilus* cells were immediately resuspended in the rescue broth used for *L. lactis* cells (Hill, C., FEMS Microbiol. Rev. 12:87-108 (1993)) and incubated for 2 hours at 42°C before they were plated on GM17 supplemented with the appropriate antibiotic.

Phage isolation. Phages φQ1 and φQ3 were recently isolated from yogurt samples whereas φQ5 and φQ6 were isolated also in our laboratory but from Mozzarella whey samples. Phages φQ1 and φQ3 were then propagated on *S. thermophilus* SMQ-119, φQ5 on SMQ-173 and φQ6 on SMQ-174. The genomic DNAs of these streptococcal phages were compared after digestion with *EcoRV* and *MboI* (Fig. 6). All four *S. thermophilus* phages had different restriction patterns (Fig. 6) and consequently they were different.

Expression of *LlaII* in *Streptococcus thermophilus*. To verify if *LlaII* system could be functional in *S. thermophilus*, the *LlaII* genes were cloned into a vector with an origin of replication functional in *L. lactis* and *S. thermophilus*. The lactic acid bacteria shuttle vector pNZ123 (2.5 kb) (DeVos, W. M., FEMS Microbiol. Rev. 46:281-295 (1987)) was selected. A 7.0-kb *EcoRI* fragment from pSRQ700 was cloned into the unique *EcoRI* site of pNZ123 (Fig. 7). The ligation mixture was electroporated directly into the phage sensitive strain *L. lactis* LM0230. Cm-resistant transformants were obtained and tested for resistance to

5 ϕ p2. A phage-resistant transformant thus obtained was named SMQ-151. The resulting pNZ123 clone containing the 7.0 kb fragment from pSRQ700 was named pSRQ707. This plasmid was electroporated into *S. thermophilus* SMQ-119 and a Cm^r -transformant was named SMQ-154. This clone was tested for resistance against two *S. thermophilus* phages (ϕ Q1 and ϕ Q3). Both phages were severely restricted on SMQ-154 since they had EOPs of 10^{-8} (Table 5).

Tabl 5. Efficiency of Plaquing of *S. thermophilus* phages on various hosts.

5	Phage / Host	EOP
	$\phi Q1$ / SMQ-119	1.0
10	$\phi Q1$ / SMQ-146	1.0
	$\phi Q1$ / SMQ-151	2.4×10^{-8}
	$\phi Q3$ / SMQ-119	1.0
	$\phi Q3$ / SMQ-151	6.1×10^{-8}
	$\phi Q5$ / SMQ-173	1.0
	$\phi Q5$ / SMQ-211	3.9×10^{-6}
	$\phi Q6$ / SMQ-174	1.0
	$\phi Q6$ / SMQ-212	1.2×10^{-5}

Plasmid pSRQ707 was also electroporated into *S. thermophilus* SMQ-173 and SMQ-174 which are commercially used for Mozzarella cheese production. Transformants were obtained for both strains, and named SMQ-211 and SMQ-212, respectively. Both transformants were tested for phage resistance. Phage Q5 had an EOP of 10^{-6} on SMQ-211 whereas ϕ Q6 and an EOP of 10^{-5} on SMQ-212 (Table 5). The phage resistance observed against Mozzarella phages was slightly weaker than with the yogurt phages, but still significant. These results show that the *LlaII* R/M system is functional in various *S. thermophilus* strains and can confer strong phage resistance in this lactic acid bacteria. This is the first report of increased phage resistance in *S. thermophilus*.

Thus, in general the present invention relates to an isolated and purified *Streptococcus thermophilus* naturally lacking in at least one phage resistance and containing recombinant DNA encoding an endonuclease from a *Lactococcus lactis* to impart the phage resistance.

Further, it relates to a method for fermenting a dairy product, the improvement which comprises using a dairy culture of *Streptococcus thermophilus* lacking in at least one phage resistance for the fermentation incorporating recombinant DNA encoding an endonuclease of *Lactococcus lactis* to impart the phage resistance.

Still further, it relates to a method of imparting phage resistance to a *Streptococcus thermophilus* which is lacking in at least one phage resistance which comprises incorporating recombinant DNA encoding an endonuclease of *Lactococcus lactis* into the *Streptococcus thermophilus* to impart the phage resistance.

The foregoing description is only illustrative of the present invention and the present invention is limited only by the hereinafter appended claims.

CLAIMS

1. A nucleic acid sequence encoding only a polypeptid , protein or enzyme which is sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* and mixtures thereof to restrict or modify a phage.

5

2. A nucleic acid sequence according to claim 1 having a nucleotide sequence selected from the group consisting of sequence s encoding the amino acid sequence of SEQUENCE ID NO 2, SEQUENCE ID NO 3 and SEQUENCE ID NO 4 and combinations thereof.

10

3. A nucleic acid sequence according to claim 1 having a nucleotide sequence as set forth in SEQ ID NO. 1 and selected from th group consisting of ORF1 (positions 97 to 948), ORF2 (positions 941 to 1747) and ORF3 (positions 1740 to 2651) and combinations thereof.

15

4. A nucleic acid sequence according to any of the preceding claims, encoding a polypeptide, protein or enzyme sufficiently duplicative of enzyme *LlaIIA* to restrict or modify a phage, said nucleic acid sequence suitably having a nucleotide sequence essentially corresponding to the nucleotide sequence set forth in ORF 1 of SEQ ID NO. 1.

20

5. A nucleic acid sequence according to any of the preceding claims encoding a polypeptide, protein or enzyme sufficiently duplicative of enzyme *LlaIIB* to restrict or modify a phage, said nucleic acid sequence suitably having a nucleotide sequence essentially corresponding to the nucleotide sequence set forth in ORF 2 of SEQ ID NO. 1.

25

6. A nucleic acid sequence according to any of the preceding claims encoding a polypeptide, protein or enzyme sufficiently duplicative of enzyme *LlaIIC* to restrict or modify a phage, said nucleic acid sequence suitably having a nucleotide sequence essentially corresponding to the nucleotide sequence set forth in ORF 3 of SEQ ID NO. 1.

30

35

7. A plasmid comprising a nucleic acid sequence according to any of the preceding claims, said plasmid not being present in its natural *Lactobacillus* host if it is a naturally occurring *Lactobacillus* plasmid.
- 5 8. An isolated plasmid comprising a nucleic acid sequence according to any of claims 1-6.
- 10 9. A recombinant plasmid comprising a nucleic acid sequence according to any of claims 1-6.
10. The plasmid of any of Claims 7-9 which is a shuttle vector comprising the nucleic acid sequence of any of claims 1-6 as inserted, said shuttle vector being for example PSA3.
- 15 11. A recombinant bacterium harboring a heterologous nucleic acid sequence according to any of claims 1-6 or a heterologous plasmid according to any of claims 7-10, said bacterium preferably being rendered phage resistant by expression of the heterologous nucleic acid sequence or plasmid.
- 20 12. A bacterium according to Claim 11 which is *Escherichia coli*.
13. A bacterium according to Claim 11 which is selected from the group of bacteria useful in food fermentation processes, in particular dairy processes for example for producing milk products such as a group e.g. consisting of *Lactobacilli* such as *Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus*.
14. A recombinant polypeptide, protein or enzyme containing a sequence of amino acids sufficiently duplicative of that set forth in a member selected from the group consisting of ORF 1, ORF 2 and ORF 3 as set forth in SEQ ID NO. 1, 2, 3 or 4 or combinations thereof exhibiting activity such that restriction or modification of a phage can be performed with said polypeptide, protein or enzyme, wherein the polypeptide, protein or enzyme has been produced from an isolated

nucleic acid sequence according to any of claims 1-6 or a plasmid according to any of claims 7-10 or a recombinant bacterium according to any of claims 11-13.

15. A recombinant polypeptide, protein or enzyme according to claim 14 substantially free of cell extracts, preferably isolated and optionally purified to a degree sufficient for use as additive in food processing.

16. A recombinant polypeptide, protein or enzyme according to Claim 14 or 15 which has an amino acid sequence as shown in any of SEQUENCE ID NO. 1, 2, 3 and 4.

17. A method of imparting phage resistance to a bacterium which is sensitive to the phage which comprises incorporating a heterologous nucleic acid sequence encoding a polypeptide, protein or enzyme sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* and mixtures thereof into the bacterium in a manner known per se for incorporating nucleic acid sequences, thereby imparting the phage resistance.

18. A method according to claim 17, wherein the nucleic acid sequence encoding the member is contained in strain *Lactococcus lactis* SMQ-17 deposited as NRRL-B-21337.

19. A method according to claim 17 or 18, wherein the bacterium is selected from a group of bacteria used in food processing, preferably in dairy processes such as Lactobacilli, e.g. the group consisting of *Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus*.

20. An improved method of fermenting a dairy product, the improvement comprising using a dairy culture selected from the group consisting of recombinant bacteria according to any of claims 11-13 and bacteria obtainable via the method of claims 17-19, in particular *Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus* comprising a heterologous nucleic acid sequence encoding an nzyme

sufficiently duplicative of a member selected from the group consisting of *LlaIIA*, *LlaIIB* and *LlaIIC* to impart phage resistance, wherein the heterologous nucleic acid sequence for the member is contained in strain *Lactococcus lactis* SMQ-17 deposited as NRRL-B-21337 in a fermentation process known per se thus achieving phage resistance.

21. An improved method for fermenting a dairy product, the improvement comprising adding a recombinant polypeptide, protein or enzyme according to any of claims 14-16 in an amount sufficient to achieve phage resistance during the fermentation process.

22. A *Streptococcus* suitable for use in food processing such as dairy processes e.g. a *Streptococcus salivarius* subsp. *thermophilus* naturally lacking in at least one phage resistance and containing a heterologous nucleic acid sequence encoding at least one endonuclease from a *Lactobacillus* e.g. a *Lactococcus lactis* and optionally said heterologous nucleic acid sequence further encoding a methyl transferase from a *Lactobacillus* e.g. a *Lactococcus lactis* to impart the phage resistance.

23. A *Streptococcus* according to Claim 22 wherein the heterologous nucleic acid sequence is derived from a natural *Lactobacillus* R/M system and thus can be a plasmid.

24. A *Streptococcus* according to Claim 22 or 23 wherein the heterologous nucleic acid sequence is derived from a naturally occurring plasmid.

25. A method of imparting phage resistance to a bacterium which is sensitive to the phage which comprises incorporating a heterologous nucleic acid sequence into the bacterium in a manner known per se for incorporating nucleic acid sequences, thereby imparting the phage resistance, said bacterium being a *Streptococcus* suitable for use in food processing such as dairy processes e.g. a *Streptococcus salivarius* subsp. *thermophilus* said bacterium naturally lacking in at least one phage resistance and said heterologous nucleic acid sequence encoding at least one endonuclease from a *Lactobacillus* e.g. a *Lactococcus lactis* and optionally said heterologous nucleic acid sequence further

encoding a methyl transferase from a *Lactobacillus* e.g. a *Lactococcus lactis* to impart the phage resistance.

26. An improved method for fermenting a dairy product, the improvement comprising using a dairy culture of *Streptococcus* according to any of claims 22-24 in a manner known per se for dairy cultures in fermentation processes and thus obtaining phage resistance.

1/10

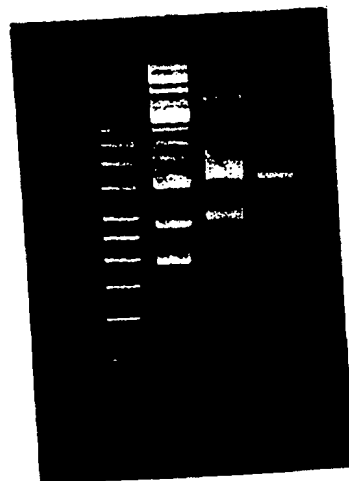


FIGURE 1

SUBSTITUTE SHEET (RULE 26)

2/10

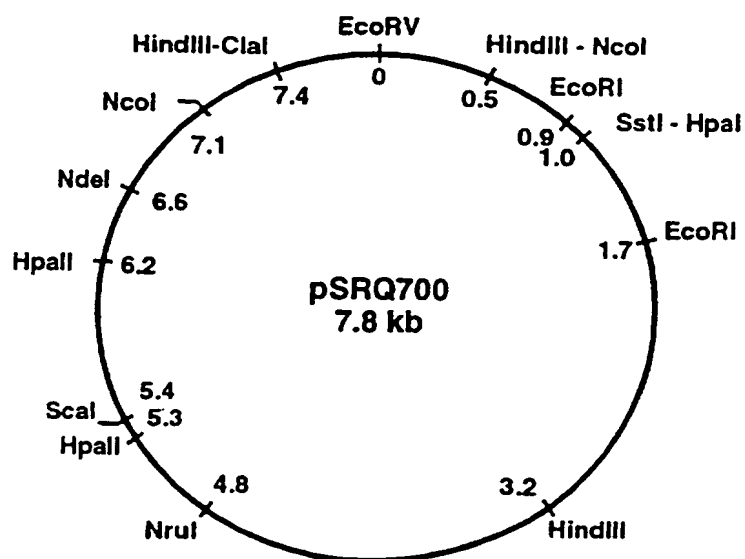


FIGURE 2

SUBSTITUTE SHEET (RULE 26)

3/10

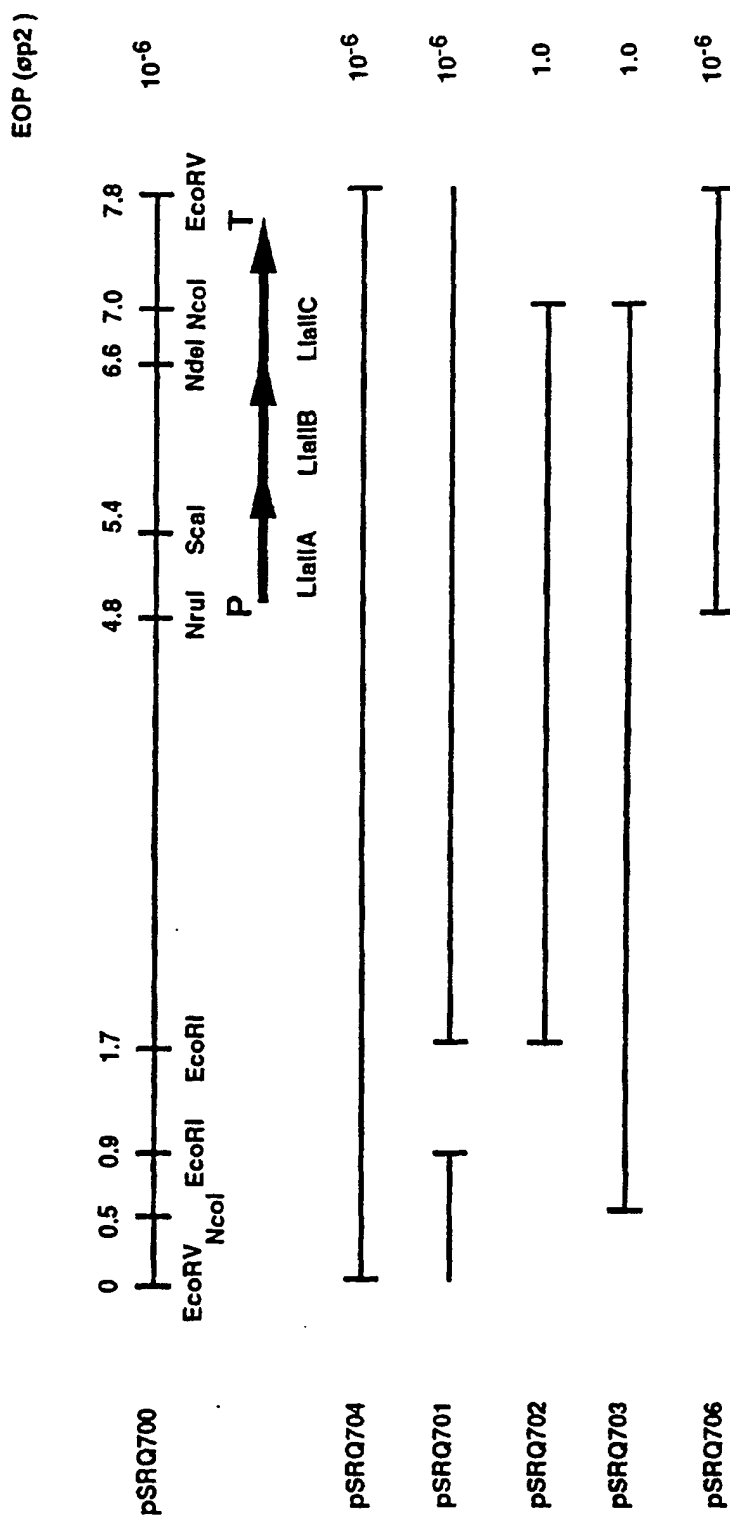


FIGURE 3

SUBSTITUTE SHEET (RULE 26)

4/10

FIGURE 4

1 CGAGCTTTCTAATGCTTAGTGCTTTAAGATTAGGATAGCACCAGCTTATTT
 51 ATTTTCCAATGAAATTAACTAGCAATTCGGGTATAATATATTTATGAAATT
 H N L
 101 TATTACAAAAAACAAGATCAACTTACGTCGTTTACTAAATGGACAGGT
 L Q K N K I N L R P F T K W T G
 151 GGGAAAAGGCAACTACTGCCACACATTCAATACCTAATGCCAGAAAAATA
 G K R Q L L P H I Q Y L H P E K Y
 201 CAATCATTTTTTCGAACCTTTTATTGCTGGTGGCGCTTGTTTTTTGAAC
 N H F F E P F I G G C A L F F E P
 251 CCGCTCTCTAAAAAGCAGTTATTAACGACTTCAATTCTGAGCTTATAAAC
 A P Q K A V I N D F N S E L I N
 301 TGTTACCGGCAGATGAAAGATAATCTGAGCAATTGATAGAAATGTTGAC
 C Y R Q H K D N P E Q L I E L L T
 351 TAATCATCAGCGGAAAAATCTAAAGAATATTTAGACTTACGTTCTT
 N H Q R E N S K E Y Y L D L R S S
 401 CTGATAGAGATGGAAGAATTGATAAGATGAGCGAAGTTGAACGCTGCTGCT
 D R D G R I D K H S E V E R A A
 451 AGAATTAATGTATATGCTACGTTGATTTTTAAATGCTTTATATCGTGTAA
 R I M Y M L R V D F N G L Y R V N
 501 TTGAAAAACCAGTTTAAATGTGCTTATGGAAGATATAAAAACTCTAAGA
 S K N Q F N V P Y G R Y K N P K I
 551 TAGTTGATAAAGAATTGATTGAAAGTATTTCCGAGTACTTGAATTAACAAT
 V D K E L I E S I S E Y L N N N
 601 TCTATTAAGATCATGAGTGGAGATTTTGAAGAACCGTTAAAGAAGCACA
 S I K I M S G D F E K A V K E A Q
 651 GGATGGAGATTTTGTTTATTTGACCCCTCCATACATTCCTACTTTCTGAAA
 D G D F V Y F D P P Y I P L S E T
 701 CTAGCGCCTTTACTTCTTATACACAGAAAGGCTTTAGCTACGAAGATCAA
 S A F T S Y T H E G F S Y E D Q
 751 GTTAGGCTAAGAGATTTGTTTCAACAGTTAGATTCAAAAAGGCTATTGCT
 V R L R D C F K Q L D S K G V F V
 801 CATGCTTTCAAAATCTTCAAGCCCTTTAGCGGAGGAATATATTAAGATT
 M L S N S S S P L A E E L Y K D F
 851 TTAAATCCATAAAAAATGAAGCTACTCGAACAAATGGGGCTAAATCATCT
 N I H K I E A T R T N G A K S S
 901 AGTGTGGAAAAATCACTGAAATCATGTAACCAATTATGGCAATTAACG
 S R G K I T E I I V T N Y G N
 M A I N E
 951 AATAAAGTATGGAGGTGTTTTAATGACAAAACCATATGAAAAAGAA
 Y K Y G G V L M T K P Y Y E K E
 1001 AAACGCAATTTCTGTTACCGAGATTCATTTAAATTAATAGAAAAAATAA
 N A I L V H A D S F K L L E K I K
 1051 ACCTGAAAGCATGGACATGATATTTGCTGACCCCTCCTTACTTTTAAAGTA
 P E S M D M I F A D P P Y F L S N
 1101 ATGGAGGAATGTCAAAATTCAGGTGGTCAAAATGTTTCTGTTGATAAAGGG
 G G H S N S G G Q I V S V D K G
 1151 GATTGGGATAAAATTTCTTCAATTGAAGAAAAACATGACTTTAAATAGACG
 D W D K I S S F E E K H D F N R R
 1201 TTGGATTAGGTTAGCAAGATTGGTTTTAAAAACCAACGGAATATTGGG
 W I R L A R L V L K P N G T I W V
 1251 TTTCGGGAAGCCCTTCAATACATATATCTGTCGGATGGCGCTGGAACAG
 S G S L H N I Y S V G H A L E Q
 1301 GAAGGTTTCAAAATCTTAATAATAATACTTGGCAAAAGACAAATCTCTGC
 E G F K I L N N I T W Q K T N P A
 1351 ACCTAATCTATCATGTCGGTACTTCACCCACTCTACAGAGACAAATTTTAT
 P N L S C R Y F T H S T E T I L W
 1401 GGGCAAGAAAGAACGATAAAAAATCTGCCATTATTATAACTATGAATTG
 A R K N D K K S R H Y Y N Y E L

SUBSTITUTE SHEET (RULE 26)

5/10

FIGURE 4 (Cont'd)

1451 ATGAAAGAGTTTAAATGACGGGAAACAAATGAAAGATGTTTGGACAGGTAG
M K E F N D G K Q H K D V W T G S

1501 TCTGACAAAAAATCAGAAAAATGGGCTGGGAAACATCCAACTCAGAAGC
L T K R S E K W A G K H P T Q K P

1551 CAGAGTATATTTTAGAACGGATAATCTTAGCTAGTACAAAGGAAAAATGAT
E Y I L E R I I L A S T K E N D

1601 TATATTTTAGACCTTTTCGTCGGAAAGTGAACACTACTGGTGTAGTAGCCAA
Y I L D P F V G S G T T G V V A X

1651 GAGATTGGGGCGTAAATTTATTGGGATTGATTCTGAGAAAGAATATCTTA
R L G R K F I G I D S E K E Y L K

1701 AAATTGCTAAAAAAGGCTAAATAAAGGAGCAACATATGAGCTTTAATAA
I A K K R L N K G A T Y G L *
H D F N N

1751 TTACATCGGTTTAGAACTCGACGATAGATTAAATGCTTTTATGGCAACAC
Y I G L E S D D R L N A F H A T L

1801 TTTCGGTAACATAAGAACTCCCGAATACTACGTGAACCTGGGAAAAAGTT
S V T N R T P E Y Y V N W E K V

1851 GAACGTGAAACACGAAAAATTTGAATTAGAACTAAATACTTTAAACTATCT
E R E T R K F E L E L N T L N Y L

1901 CATTTGGAAAGAAGATATTTATAGTGAAGCACTTGAACATTTTACCAATC
I G K E D I Y S E A L E L F T N Q

1951 AACCTGAATTGCTTAAAGCTATTCCTAGTTTGAATTGCTAGTAGAGATACA
P E L L K A I P S L I A S R D T

2001 TCTTTAGATAATCAACATTGACGAAAAATGATGATAGTGTTTGAACA
S L D I L N I D E N D D H S F E Q

2051 ACTTAACCTTCTTGTATCGACGAAAAATGTTATCGCTGATATGATGACT
L N F L V I D E N C I A D Y V D F

2101 TTATTAACCAAGGAGGTTTACTAGATTTCCTACAGAATAAAGCAAAAAAGT
I N Q A G L L D F L Q N K A K R

2151 TCTCTGGTAGACTATGTTATGGTGTGGAAGCAGGGCTTGAAGCAATGC
S L V D Y V Y G V E A G L D S N A

2201 TCGAAAAACCGAAGCGGTACAACCATGGAAGGGAATTTAGAACGTACTC
R K N R S G T T H E G I L E R T V

2251 TTTCAAAAATAGCTCAAGAGAAAGGGCTTGAATGGAAGCCACAGGCAACC
S K I A Q E K G L E W K P Q A T

2301 GCTTCTTTTATCAAGTCTCAATGGGACATAGAAGTCCTGTAGATAAATC
A S F I K S Q W D I E V P V D K S

2351 AAAAGACGCTTTGATGACGAGTTTACTCTGGTGGCTCAATAAGGTTT
K R R F D A A V Y S R A L N K V W

2401 GGCTCATAGAAACAAATTAATAAGGCGGTGGAGGAAGTAAACTCAAAGCA
L I E T N Y Y G G G G S K L K A

2451 GTTGCTGGAGAAATTCAGAAATGAGTCAGTTTGTAAAAACATCAAAGA
V A G E F T E L S Q F V K T S K D

2501 TAATGTTGAATTTGTTATGGTTAACAGACGGCCAAAGGTTGGAATTTTCCC
N V E F V W V T D G Q G W K F S R

2551 GCTTACCACTTGCAAGCTTTTCGGACACATCGATAACGTTTTCATCTA
L P L A E A F G H I D N V F N L

2601 ACCATGTTGAAAGAAGGTTTCTTATCTGATTTATTTCGAAAAAGAAATTTA
T M L K E G F L S D L F E K E I *

2651 AAAAGACAGAGAAATCTCTGCTTTTAAATTTCAATTCTCTCTCTGCT
AGCTATACTTTCCAAAAACCTGAAAAACGGTTCTGTTGCAATTTGATG
TGGGTCGGAACCTACTACTATATCATGAGAAATGAAGATTAAAGTTGAA
ACAAAAAACAGATTATTTTAAATGTAATCTGTTTGTGTTGGGCTGA
TTTATATCACCAATTCTATGTTTCAGAAAAATGGTCAATTTCTGGACACTC
TTCTTTGTTATTAAACTCTCAAAATCATTTACATTTATGTTTCATTAA
CCGTAATTTATCTATGTTTCAATTTATAGATATC

SUBSTITUTE SHEET (RULE 26)

6/10

[illegible]

FIGURE 5

7/10

Fig. 5B)

	MotifII	
M.LlaIIB	MAINEYKYGVLMTKPYEKENA--ILVHADSFKLEKIKPESMDMIFADPPYFL-SNGGMSNSGGQIVSVDKGDWDK	75
M.DpnA	MKNNEYKYGVLMTKPYNNKMM--ILVHSDTFKFLSKMKPESMDMIFADPPYFL-SNGGISNSGGQIVSVDKGDWDK	75
M.MboC	MRIKPYFESDDKFNIVQNCIDFMSHFQDNIIMIFADPPYFL-SNDGLTFKNSIIQSVNKGWDK	66
M.HinfI	MMKENINDFL-NTILKG-DCIEKLKTIIPNESIDLIFADPPYPMQTEGKLLRTNGDEFSGVDDEWDK	64
M.LlaIIB	ISSFEKHDPNRRWIRLARLVLPNGTIWVSGSLHNIYSVGMALQEKGFKILNNITWQKTNPAPNLSCRYFTHSTET	152
M.DpnA	ISSFEKHDPNRRWIRLARLVLPNGTIWVSGSLHNIYSVGMALQEKGFKILNNITWQKTNPAPNLSCRYFTHSTET	152
M.MboC	NDNEASIYNFNHETIAQARQLLDKNGTIWISGTHHNIFTVGQVLKENNFKILNIITWEKPNPPNFSCRYFTYSSEW	143
M.HinfI	FNDFVEYDSFCELMWKECKRILKSTGSIWVIGSFQNIYRIGYIMQNLDPWILNDVIWKNTPVPNFGGTRPCNAHET	141
M.LlaIIB	ILWARKNDKKSRIHYNYELMKEFNDGKQMKDVWTGSLTKKSEK---AGK--HPTQKPEYILERIILASTKENDYIL	224
M.DpnA	ILWARKNDKKSRIHYNYELMKELNDGKQMKDVWTGSLTKKSEK---AGK--HPTQKPEYILERIILASTKENDYIL	224
M.MboC	I IWARKH-SKIPHYFNVDLMKKLNGDKQKDIWRLPAVGSWEKT---QCK--HPTQKPLGLLSRIILSSTQKDDLIL	214
M.HinfI	MLWCSKC-KKNKFTFNKTMKHLNQEKQERSVWSLSLCTGKERIKDEEGKKAHSTQKPESLLYKVILSSSKPNDVVL	217
	MotifI	
M.LlaIIB	DPFVGSQTTGVVAKRIGRKFIGIDSEKEYLKI AKKRLNKGATYGL	269
M.DpnA	DPFVGSQTTGVVAKRIGRRFIGIDAEKEYLKI AKRLEAENETN	268
M.MboC	DPFVGSQTTGIAGVLLDRNYIGIEQELEFLELSKRRYHEITPVLKNEFKQKIRKQISAI	273
M.HinfI	DPFFGTGTTGAVAKALGRNYIGIEREQKYIDVAEKRLREIKPNPNDIELLSLEIKPKVPMKTLIEADFL	287

FIGURE 5 (Cont'd)

8/10

Fig. 5C)

R.LlaII	MDFNNYIGLESDDRLNAFMATLSVTNRTPEYVNMKEVERETRKFELNLTNLNLYLGKEDIYSEALEFTNQPELLKAI	79
R.DpnII	MKQTRNFDEWLSMTDVTADWTYYTDFPQVYKNVSSIKVALNIMNSLIGSKNIQEDFLDYQNYPEILKVV	71
R.MboI	MKLAFFDDFLNSMSETNTTLDYFTDFDKVKNVAQIEIHLNQLNLYLLGKDDLKQAVYDLYAECNPFSL	69
R.LlaII	PSLIASRDTSLDILNIDENDDDMSFEQLNPLVIDENCIAIADYVDFINQAGLLDFLQNKAKRSLVDYVYGVEAGLDSNAR	156
R.DpnII	PLLIARLRTDIIVK-DPIKDFYD----FSKRNYSIEEYTMFLEKSGIFDQLLQNHLSVNLVDYVVTGVEVGMDTNCR	143
R.MboI	EILIAVRKKE-QKKSLEKGGQVVTLSYF-----QSADKIIDFLNNTGLADVFRDKNIKNLVDYVFGIEVGLDTNAR	140
R.LlaII	KNRSCTTMEGILERTVSKIAQEKGLEWKQATASFIKSQWDIEVPV---DKSKRRPDAAVYSRALNKVWLIEVTNYYG	230
R.DpnII	KNRTGDAMENIVQSYLEAEGYILGENLFKEIEQNEIEIFSVDLISAITNDGNTVKRDFVI--KNEQVLYLIEVNFYS	219
R.MboI	KNRGGDNM-----SKAVQLLFDNADIYKKKEVRNTIFT---DIE-SL-----GADVQKQDFVI--KTKRKYVIETNYYN	204
R.LlaII	GGGSKLKAVAGEFTLSQFVKTSKDNVEFVWVTDGQGWKFSRLPLAEAFGHIDNVFNLTMLKEGFLSDLFEKEI	304
R.DpnII	GGGSKLNETARSYKMIAEETKAI-PNVEFMWITDQGWYKAKNNLRETDFDILPFLYNINDLEHNILKNLK	288
R.MboI	SGGSKLNEVARAYTDVAPKINQYSQ-YEPVWITDQGWKTAKNKLQEAETHIPSVYNLYTL-HGFIEQLNSEGVIKDW	280

FIGURE 5 (Cont'd)

9/10



FIGURE 6

SUBSTITUTE SHEET (RULE 26)

10/10

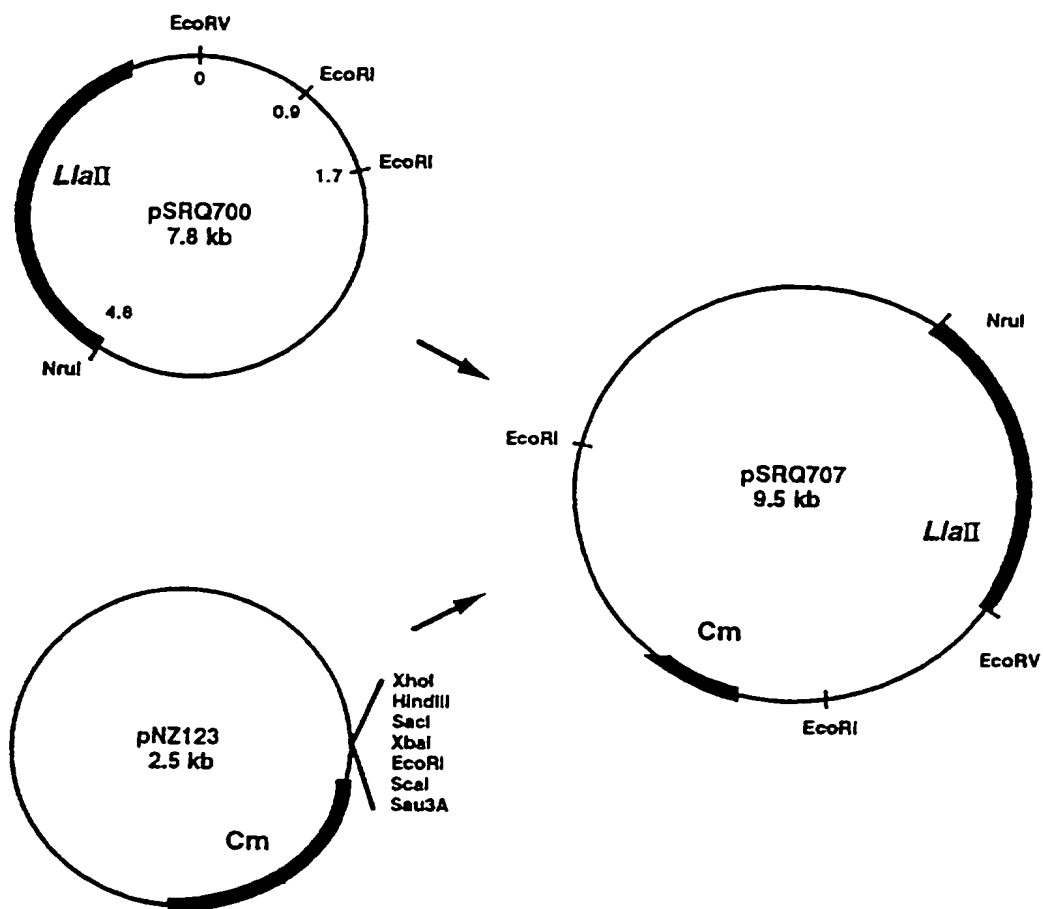


FIGURE 7

SUBSTITUTE SHEET (RULE 26)

9

10



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/31, 15/54, 15/55, C07K 14/315, C12N 9/22, 9/10, 15/75, 1/20, A23C 9/123 // C12R 1:46	A3	(11) International Publication Number: WO 96/21017 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/NL95/00448 (22) International Filing Date: 29 December 1995 (29.12.95) (30) Priority Data: 08/366,480 30 December 1994 (30.12.94) US 08/424,641 19 April 1995 (19.04.95) US (71) Applicant (for all designated States except US): QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): MOINEAU, Sylvain [CA/CA]; 3605 Place du Houx, Charlesbourg, Quebec G1G 3G9 (CA). WALKER, Shirley, A. [US/US]; Apartment 16, 5416 Portree Place, Raleigh, NC 27606 (US). VEDAMUTHU, Ebenezer, R. [US/US]; 2710 5th Avenue N.W., Rochester, MN 55901 (US). VANDENBERGH, Peter, A. [US/US]; 4414 Meadowcreek Circle, Sarasota, FL 33583 (US). (74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 21 November 1996 (21.11.96)
(54) Title: ISOLATED DNA ENCODING ENZYME FOR PHAGE RESISTANCE (57) Abstract An isolated DNA of a <i>Lactococcus lactis</i> showing a SEQ ID NO:1 encoding a restriction and two modification enzymes (R/M SEQ ID NO:2, 3 and 4). The isolated DNA is used to transform sensitive dairy cultures, such as <i>Lactococcus lactis</i> and <i>Streptococcus thermophilus</i> , to provide phage resistance. <i>Escherichia coli</i> can be used to produce endonucleases.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 95/00448

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N15/54 C12N15/55 C07K14/315 C12N9/22
 C12N9/10 C12N15/75 C12N1/20 A23C9/123 //C12R1:46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NUCLEIC ACIDS RESEARCH, vol. 21, no. 10, 25 May 1993, pages 2309-2313, XP002005772 UENO, T. ET AL.: "Gene structure and expression of the MboI restriction-modification system" cited in the application Figure 3</p> <p>---</p> <p>-/--</p>	<p>1,4-9, 11,12, 14,15</p>

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 September 1996

Date of mailing of the international search report

15. 10. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Alt, G

INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/NL 95/00448

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 82, July 1985, pages 4468-4472, XP002005773 MANNARELLI, B.M. ET AL.: "Nucleotide sequence of the Dpn II DNA methylase gene of Streptococcus pneumoniae and its relationship to the dam gene of Escherichia coli" Figure 5 ---	1,4-9, 11,14,15
P,X	GENE, vol. 157, 22 May 1995, pages 13-18, XP002005774 NYENGAARD, N. ET AL.: "Restriction-modification system in Lactococcus lactis" page 17 ---	1-9, 11-17,19
O,X	Third New England Biolabs Workshop on Biological DNA Modification Vilnius, Lithuania, 22-28 May 1994 ---	1-9, 11-17,19
X	GENE, vol. 136, 1993, pages 371-372, XP002005775 NYENGAARD, N. ET AL.: "LlaAI and LlaBI, two type-II restriction endonucleases from Lactococcus lactis subsp. cremoris W9 and W56 recognizing, respectively, 5'-/GATC-3' and 5'-C/TRYAG-3'" whole document ---	14,15
X	EP,A,0 316 677 (MILES INC.) 24 May 1989 page 10, last paragraph; page 11; Example 6 ---	22-26
Y	BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 19, no. 3, August 1991, pages 675-681, XP002005776 KLAENHAMMER, T.R.: "Development of bacteriophage-resistant strains of lactic acid bacteria" page 677, right-hand column, last paragraph - page 678, left-hand column, third paragraph ---	20,21
Y	US,A,4 883 756 (NORTH CAROLINA STATE UNIVERSITY) 28 November 1989 column 4, lines 12-22 --- -/--	20,21

Form PCT/ISA:210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 95/00448

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, no. 1, January 1993, pages 197-202, XP002005777 MOINEAU, S. ET AL.: "Restriction/modification systems and restriction endonucleases are more effective on lactococcal bacteriophages that have emerged recently in the dairy industry" whole document	1-21
A	--- FEMS MICROBIOLOGY REVIEWS, vol. 87, 1990, pages 61-78, XP002014350 MERCENIER, A.: "Molecular genetics of Streptococcus thermophilus" pages 67-73	22-26
A	--- LAIT, vol. 73, 1993, pages 175-180, XP002014351 MOLLET, B. ET AL.: "Molecular genetics in Streptococcus thermophilus: from transformation to gene expression" whole document	22-26
P,X	--- APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 6, June 1995, pages 2193-2202, XP002005778 MOINEAU, S. ET AL.: "Cloning and sequencing of LlaII restriction/modification genes from Lactococcus lactis and relatedness of this system to the Streptococcus pneumoniae DpnII system" whole document	1-21
P,X	--- APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 7, July 1995, pages 2461-2466, XP002014352 MOINEAU, S. ET AL.: "Expression of a Lactococcus lactis phage resistance mechanism by Streptococcus thermophilus" whole document	22-26

INTERNATIONAL SEARCH REPORT

I. national application No.

PCT/NL95/00448

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-21
- claims 22-26

* see continuation-sheet PCT/ISA/210 *

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

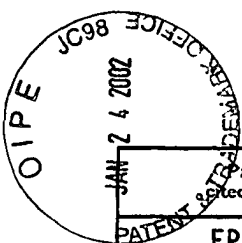
Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

claims 1-21: Nucleic acid fragment encoding LlaIIA, LlaIIB, LlaIIC, plasmid containing said fragment, recombinant bacterium containing said plasmid, polypeptide encoded by said fragment, methods of imparting phage resistance and improving a dairy product by using said nucleic acid, said bacteria or said polypeptide

claims 22-26: Streptococcus containing nucleic acid encoding at least one endonuclease from Lactobacilli and its use in a method of imparting phage resistance and improving a dairy product



INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 95/00448

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0316677	24-05-89	AU-A- 2503588	18-05-89
US-A-4883756	28-11-89	NONE	

